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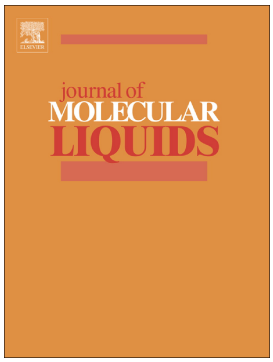

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Quaternary ammonium surfactants derived from leucine and methionine: novel challenging surface active molecules with antimicrobial activity

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Abstract

Surfactants are versatile excipients, which are commonly employed in several pharmaceutical formulations as emulsifiers, stabilizing or solubilizing agents. In this field, current research is aimed at the design or discovery of amphiphilic molecules with improved characteristics in terms of safety (toxicological profile) and biological activities (e.g. antimicrobial activity, interaction with biomembranes). Quaternary ammonium surfactants derived from amino acids are one such class of compounds with significant potential for pharmaceuticals. In this work, quaternary ammonium surfactants derived from the amino acids leucine and methionine and esterified with fatty acid of different lengths (C10, C12 and C14) were synthesized. These amphiphiles were characterized in terms of critical micelle concentration (CMC) by tensiometry and conductivity measurements, cytotoxicity (MTS and LDH assay on Caco-2 and Calu-3 cell lines) and antimicrobial activity on selected Gram-positive (*S. aureus and E. faecalis*), Gram-negative (*E. coli and P. aeruginosa*) and the fungus *C. albicans*. The derivatives bearing C12 and C14 chains displayed CMC, EC50 (from cytotoxicity assays) and minimum inhibition concentration (MIC) values that are comparable to those of benzalkonium chloride (used as reference), especially towards Gram-positive and *C. albicans*. On the contrary, derivatives bearing C10 chains have higher CMC, EC50 and MIC values, highlighting the crucial role of the hydrophobic tails in determining both physicochemical and biological properties. The C12-C14 synthesized derivatives may represent a promising alternative to benzalkonium chloride as surface active molecules and antimicrobial agents.

Keywords: hydrophobic tail, amino acid, critical micelle concentration (CMC), surface tension, cytotoxicity, minimum inhibitory concentration (MIC)**.**

1. Introduction

Surfactants are amphiphilic molecules with several applications (e.g. emulsifying, stabilizing and solubilizing agents) in different fields, including pharmaceuticals, cosmetics and food industry [1]. Cationic surfactants in particular are well known for their antimicrobial activity and are currently employed as disinfectants, antiseptics and preservative agents [2, 3]. Several classes of cationic surfactants have been explored and characterised in terms of surface activity and antimicrobial properties [4–7]. Positively-charged amphiphilic molecules can be pH-dependent (such as alkylated or ethoxylated amines and guanidines) or pH-independent (e.g. quaternary ammonium salts or imidazole-based amphiphiles), according to the functional group [8] [9] [10]. The most investigated and commercially-available cationic surfactants are amphiphiles based on quaternary ammonium salts [11] [12] [13]. Structurally, they possess a pH-independent positive charge since nitrogen is covalently linked to four different alky or aryl groups. Several chemical modifications can be performed, giving a plethora of compounds with different chemical-physical and biological properties according to the length and the nature of each substituent on the nitrogen atom [11, 14]. Quaternary ammonium salts, such as benzalkonium chloride, or cetrimide, are common excipients in ophthalmic, nasal, auricular or skin topical formulations as preservatives [15–17]. Indeed, the biocidal activity of these compounds towards most bacteria and fungi has been known since 1930s [18]. It is ascribed to the adsorption and penetration of the alkylammonium cation in the bacterial wall and to the consequent cytoplasmic membrane disruption, which causes leakage of intracellular low molecular weight material, degradation of proteins and nucleic acids, and activation of an autolytic process [19].

It is possible to prepare quaternary ammonium surfactants starting from esters of amino acids with fatty acids, via alkylation of the nitrogen in the amine group. Surfactants composed of cationic amino acids (arginine, histidine, lysine) have been widely investigated [20] [21] [22][23], but quaternary ammonium salts derived from amino acids possessing amphiphilic characteristics have not been reported.

The aim of the present work was to synthesize and characterise quaternary ammonium surfactants derived from the amino acid leucine and methionine esters with fatty acid of different lengths (C10, C12 and C14). Even though quaternary ammonium surfactants are used extensively, concerns regarding their organism- and environment-related toxicity remain. Indeed, the application of these compounds in pharmaceutical and cosmetics fields is limited to topical formulations and they are not approved for internal use due to toxicological concerns. Amino acid-based surfactants have shown mild toxicological profiles on cell lines and quaternary ammonium surfactants based on amino acids could also show favourable biological properties. To this end, in this work we conducted cytotoxicity studies on human epithelial cell lines, Caco-2 (intestinal) and Calu-3 (airway), in addition to examining antimicrobial properties of these materials on select Gram-positive and Gram-negative bacteria and fungus *Candida albicans*.

2. Materials and methods

2.1 Materials

L-leucine (LEU), L-methionine (MET), 1-decanol, p-toluenesulfonic acid (p-TSA), toluene anhydrous (99.8%) and iodomethane purum ≥ 99.0%, were purchased from Sigma-Aldrich (Milan, Italy). 1-dodecanol and 1-tetradecanol were purchased from DPI (Lancaster, SC, USA). Sodium carbonate and potassium carbonate were purchased from Carlo Erba (Milan, Italy). All used solvents (acetone, ethyl acetate, diethyl ether, methanol) were of analytical grade. Ultrapure water was obtained with a laboratory deionizer (Osmo lab UPW2, Gamma 3, IT). Benzalkonium chloride (BAC; purity ≥ 95.0%) composed of benzyldimethyldodecylammonium chloride (~70%) and benzyldimethyltetradecylammonium chloride (~30%) (from HPLC area % according to the manufacturer specifications, average molecular weight 348.41 Da) was purchased from Sigma-Aldrich (Milan, Italy). Dulbecco's Modified Eagles Medium (DMEM), foetal bovine serum (FBS), antibiotic/antimycotic solution (10–12,000 U/ml penicillin, 10–12 mg/ml streptomycin, 25–30 μg/ml amphotericin B) and trypsin–EDTA solution (2.5 mg/ml trypsin, 0.2 mg/ml EDTA) were purchased from Sigma-Aldrich (Poole, UK). MTS reagent, 3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethony phenol)-2-(4-sulphophenyl)-2H-tetrazolium (commercially known as CellTiter96® AQ_{ueous} One Solution Cell Proliferation Assay), was purchased from Promega (USA). PierceTM LDH assay kit was purchased from ThermoScientific. Tissue culture flasks (75 cm^3 with ventilated caps) were purchased from Costar.

2.2 Methods

2.2.1 Synthesis of quaternary ammonium amino acid-based surfactants

The methyl quaternary ammonium salts of L-leucine and L-methionine, esterified with fatty acids of different lengths (C10, C12 and C14), were prepared via two steps: firstly, the coupling between the free carboxyl group of the amino acid and the corresponding alcohol were performed, giving the *O*-acyl amino acid derivatives. Then, the quaternary ammonium salts were obtained by methylation of the primary amine of the *O*-acyl amino acid. The synthetic approach is reported in scheme 1.

Synthesis of O-acyl LEU and O-acyl MET derivatives

In an anhydrous flask, 2 grams of L-leucine or L-methionine were dispersed in anhydrous toluene (40 ml), followed by the addition of the appropriate alcohol (1-decanol, 1-dodecanol, 1-tetradecanol; molar ratio 1:1.1). Once this process was completed, the required amount of p-toluenesulfonic acid (molar ratio 1:1.2) was gradually added and the reaction was refluxed for 4 hours using a Dean-Stark apparatus. Subsequently the organic part (toluene) was evaporated under vacuum, obtaining an oil as a crude product. The oil was dissolved in ethyl acetate and the excess of p-toluenesulfonic acid was extracted using Na_2CO_3. The organic phase was collected and then dehydrated with anhydrous sodium sulfate, followed by evaporation under vacuum, obtaining the compound as an oil [24, 25].

Synthesis of N, N, N trimethyl O-acyl LEU and MET derivatives

The synthesized *O*-acyl leucine or *O*-acyl methionine oils were firstly solubilized in approximately 100 mL of acetone, followed by dropwise addition of K_2CO_3 (molar ratio 1:4) and CH_3I (molar ratio 1:4); the mixture was stirred for 24 hours at room temperature. After this, the reaction was cooled down to 4 °C and filtered to remove K_2CO_3. The organic solvent was evaporated under vacuum. The quaternary ammonium salt was separated from the crude product by precipitation in ethyl ether. After filtration and drying, the final products were purified from ethanol by repeated crystallization [26].

Chemical structures of all synthesized surfactants and relative abbreviations are reported in the supplementary materials ST1. Chemical identification of the surfactants was performed by ^{1}HNMR

using DMSO as deuterated solvent and electrospray (ESI) mass analysis. The NMR analyses were recorded on a Varian EM-400 MHz spectrometer. ESI mass spectra were recorded on an apparatus (HP 1100 LC/MSD, Agilent), equipped with a single quadrupole detector. The sample was analysed after direct injection in positive mode. ^{1}H NMR chemical identification of the signals and mass spectra are reported in the supplementary materials ST1 and ST2.

2.2.2 Surface tension measurements

The tensiometric analyses were carried out with a DCA-100 tensiometer (First Ten Angstrom, USA) using the De Nouy ring method. Different concentrations for each surfactant, dissolved in ultrapure water, were tested as a function of their ability to decrease air-water surface tension at 25 °C. Each recorded surface tension value was the mean of three consecutive measurements. The critical micelle concentration (CMC, i.e. the minimum concentration at which surfactants aggregate to form micelles) and the surface tension at CMC (γCMC) were determined by the 'breakpoint' of the plot surface tension-concentration of surfactant. The breakpoint is the point of intersection of the two lines fitting the experimental data by a linear regression model (Prism version 5.0, GraphPad Inc., USA).

From tensiometric measurements, other two parameters related to the surface behaviour of surfactants were calculated. The 'surface excess concentration', Γ_{max}, which is a measure of how many surfactant molecules are adsorbed at the air-water interface, was calculated using the Gibbs equation [27]:

$$\Gamma_{MAX} = \frac{1}{2.303\ nRT}\left(\frac{\delta\gamma}{\delta \log C}\right)$$

where: R is the gas constant (8.314 J/mol K); T is the absolute temperature (K); n is equal to 1 for single chain ionic surfactants displaying only one ionisable group, dγ/d log c can be calculated from the slope of the straight section before the CMC in the plot surface tension-concentration of surfactant.

From Γ_{max}, the area occupied by a single molecule of surfactant at the water-air (As) is derived through the equation [27]:

$$A_{min} = \frac{10^{18}}{\mathrm{N}\Gamma_{MAX}}$$

All analyses were performed in triplicates.

2.2.3 Conductometric analysis

Specific conductivities (μS/cm) of different concentrations of each surfactant were measured using a microCM 2200 (CRISON, Spain) at 25 °C. For each surfactant solution, electrical conductivity was measured and plotted against the concentration. Each analysis was repeated 3 times. CMC was determined by the interception of the two lines fitting the experimental data.

2.2.4 Cytotoxicity studies (MTS and LDH assays)

Caco-2 cells (from the European Collection of Cell Cultures) and Calu-3 cells (from the American Type Culture Collection; ATCC) were cultured in DMEM supplemented with 10% of FBS and 1% of antibiotic-antimycotic mixture and incubated at 5% CO_2 and 37 °C.

Two cytotoxicity assays (MTS and LDH) were performed to investigate the cytotoxicity profiles of the quaternarium ammonium surfactants derived from leucine and methionine in comparison to the commercial BAC. The effect on cell viability (%) of the synthesized surfactants at different concentrations was evaluated through the MTS colorimetric assay. LDH was performed to assess the effect of surfactants on membrane disruption, as a sensitive and relevant assay given the materials.

The assays were conducted as reported in our previous publication [28]. The EC_{50} values from the MTS assay (concentration of surfactant that causes 50% cell death) and the LDH assay (concentration of surfactants that causes 50% LDH release from cells) were calculated using a nonlinear regression model (TableCurve 2D v5.01) as follows [29]:

$$Y = \text{BOTTOM} + \frac{\text{Top} - \text{Bottom}}{1 + 10^{(\text{LogEC50} - \text{x})\ \text{Hill Slope}}}$$

where Top and Bottom are plateaux in the units of the Y axis.

2.2.5 Antimicrobial assay and MIC determination

The antimicrobial activity of the synthesized quaternary ammonium surfactants was evaluated against four bacterial and one fungal species. Bacteria included two Gram-positives, *Staphylococcus aureus* ATCC1 25923 and *Enterococcus faecalis* ATCC 29212, and two Gram-negatives, namely *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853. The fungus was the yeast *Candida albicans* ATCC24433.

Bacterial strains were cultured overnight at 35°-36 °C in blood agar plates. Minimum inhibitory concentrations (MIC) were determined by the broth microdilution method as recommended by EUCAST (European Committee on Antimicrobial Susceptibility Testing). Twofold serial dilutions of each compound were prepared in 96-well plates, starting from 256 mg/L in Cation Adjusted Mueller Hinton Broth. An equal volume of bacterial inoculum (10^6 CFU/mL), obtained by direct colony suspension of an overnight culture, was added to each well of the microtiter plate containing 0.1 mL of the serially diluted test molecules. After incubation for 18-24 h at 35 °C in normal atmosphere, MICs were determined as the lowest concentration of compound able to inhibit the growth of the microorganisms. The same protocol with proper modification was followed to determine MICs against the *Candida albicans*. All tests were done in triplicates. Minimum Bactericidal Concentration (MBC) was subsequently determined as recommended by the EUCAST.

2.2.6 Statistical analyses

All statistical comparisons were made through Prism version 5.0 software (GraphPad Inc., USA). The correlation coefficients (r) were calculated for each data set by Pearson correlation analysis. P values <0.05 were considered as statistically significant.

3. Results

3.1 Synthesis and purification of amino acid-based quaternary ammonium surfactants

The quaternary ammonium surfactants derived from leucine and methionine were obtained by a two-step synthetic approach with high yield (> 80%, ST1). All synthesized compounds have been obtained with a high grade of purity as assessed by ^{1}H-NMR and mass analysis. NMR spectra do not show any appreciable signal related to impurities, as free alchools, or by-products of the synthesis, as mono-or di- *N*-methylated compounds. ESI mass spectra clearly display only one molecular ion (M+, *m/z*) for each synthesized surfactants, corresponding to the mass of quaternary ammonium cation. In the case of BAC, the presence of two molecular ions at 304.4 *m/z* and 332.4

m/z confirmed the presence of benzyldimethyldodecylammonium chloride (C12) and benzyldimethyltetradecylammonium (C14) chloride, as declared by the manufacturer (ST2).

3.2 Surface activity and CMC determination

Figure 1 shows the effect of the ammonium quaternary surfactants derived from leucine and methionine in decreasing air-water surface tension. The traces have similar profiles between surfactants with the same hydrophobic chain (C10, C12 or C14) and a different polar head (leucine or methionine), reaching a constant value of surface tension at lower concentrations for the derivatives with longer hydrocarbon chain. Surface properties of these amphiphiles can be compared to each other and in relation to BAC through the determination of surface parameters from the surface tension *vs* concentration plot (Figure 1). Particularly, leucine-derived surfactants showed a slightly lower CMC value than methionine-derived surfactants at any length of the hydrocarbon chain, although this difference was not statistically significant (ANOVA analysis followed by Tukey's multiple comparison, $P < 0.05$). Moreover, the derivatives bearing a C12 hydrocarbon chain (TMOL LEU and TMOL MET) displayed a CMC comparable to that of BAC, which is composed of mostly 70% C12 *N*-alkyl group, according to the manufacturer specifications (Table 1). Moreover, the elongation of the hydrocarbon chain had a marked effect on CMC values, moving from around 3 mM for C10 derivatives to around 0.4 mM for C14 derivatives. On the other hand, γCMC values (surface tension at the CMC), remained largely constant and seemed not to be directly affected by the different amino acid polar head and length of the hydrocarbon chain. Moreover, these values are comparable to those obtained with BAC, confirming the effectiveness of quaternary ammonium amino acid-based surfactants to be adsorbed to the interface and to act as surface active molecules.

The tendency of these compounds to be adsorbed at air-water interface is confirmed by Γ_{max} and A_{min} values (Table 1). These values were found to be dependent both on the length of the hydrophobic chain and the different amino acid (polar head) as already reported for other amphiphiles [28,30,31]. Indeed, the Γmax values decrease, and consequently A_{min} values increases, in the homologous series of amphiphiles with the same amino acid, as a function of the length of the hydrophobic chain (with the exception of TMOM MET). At the same time, LEU surfactants have a higher adsorption at the air-water interface, as resulted from higher Γmax and lower A_{min} values than MET surfactants [32].

Conductimetry is a technique commonly employed to determine CMC for electrically charged surfactants since they behave as electrolytes, thereby affecting the conductivity of the solution as a function of concentration. Figure 2 shows the variation of the electrical conductivity over concentration for synthesized quaternary ammonium surfactants derived from leucine or methionine, in comparison to BAC. Data show an evident inflection point, which is recognised as the CMC, due to the larger electrical conductivity increase of the solution when surfactants are present as unimers compared to micelles. CMC values, calculated from conductivity measurements, were comparable to those from tensiometry (Table 1).

3.3 Cytotoxicity studies

The cytotoxicity profiles of quaternary ammonium surfactants were determined *in vitro* on the selected cell lines, Caco-2 and Calu-3, utilising MTS and LDH assays that have different mechanisms of action. Figure 3 and figure 4 show the variation of cell viability (%) and LDH release (%) over different surfactant concentration for the MTS and LDH assay, respectively. EC_{50} values were then calculated and reported in Table 2. As for CMC, EC_{50} values were found to be dependent on the hydrophobicity of the surfactant, which is mainly dependent on the length of the hydrocarbon chain. Indeed, as hydrophobicity increases, CMC and EC_{50} values decrease. Generally, slightly higher values were calculated from LDH assay, while no remarkable differences in cytotoxicity were observed between the two selected cell lines. Yet, the calculated EC_{50} values for BAC on both cell lines were comparable to those determined from the amino acid ammonium quaternary surfactants with a C12 or a C14 hydrocarbon chain. These values were also in the range of cytotoxic concentrations previously determined for BAC in other mammalian epithelial cells as reported in the literature [33,34].

3.4 Antimicrobial activity

The antimicrobial activity of the synthesized quaternary ammonium surfactants derived from leucine and methionine was assessed by the determination of the MIC (minimum concentration of compound needed to inhibit bacterial growth) and MBC (minimum bactericidal concentration) against Gram-positive (*S. aureus* and *E. faecalis*) and Gram-negative bacterial species (*E. coli* and *P. aeruginosa*), as well as against *Candida albicans*, in comparison to the reference compound, BAC. Gram-positive bacteria were the most susceptible both towards BAC and quaternary ammonium surfactants derived from amino acids. The derivatives with a C12 and C14 chain,

regardless of the polar head (leucine or methionine), gave MIC values comparable to BAC. As expected, Gram-negative bacteria were less susceptible towards both BAC and the synthesized surfactants, most likely due to the presence of the outer membrane that constitutes an important barrier to the penetration of many small-sized molecules into the periplasmic space, by virtue of its asymmetric composition with the outer layer composed of lipopolysaccharides [35] [36]. Regarding the pathogenic fungus *Candida albicans*, MICs of amino acid-derived quaternary ammonium surfactants were comparable to BAC and, similarly to bacterial species, lower MICs were obtained for surfactants with C12 and C14 chains (Table 3).

Overall, no notable differences were observed in terms of MIC between surfactants with the same polar head (leucine or methionine) and different length of the hydrophobic chain, suggesting that the amino acid does not influence the antimicrobial activity, independently from the species. On the other hand, the length of the hydrocarbon chain has a positive effect, since derivatives with a C12 or a C14 hydrocarbon chain length are more active than the C10 chain length derivative.

The lowest antibacterial activity both for BAC and the synthesized quaternary ammonium amphiphiles derived from amino acids was found towards the gram-negative *P. aeruginosa*.

According to the obtained MBC values, the antimicrobial activity of all the synthesized amino acid-derived quaternary ammonium surfactants can be categorized as bactericidal (MBC/MIC ratios were always lower than 4) [37].

The selectivity index (EC_{50}/MIC), which can be considered as a measure of the safety of the surfactants, was also calculated. The higher this index is, the more antimicrobial activity can be exploited at non cytotoxic concentrations. Generally, a compound with a selectivity index >10 can be considered as exerting a selective antimicrobial activity in comparison to cell toxicity. As shown in Table 4, selectivity index values are close to or higher than 10 for all quaternary ammonium amino acid-based surfactants with respect to Gram-positive bacteria (*S. aureus* and *E. faecalis*), especially for Caco-2 cells. The lower values (<1) were calculated for the Gram-negative *P. aeruginosa*. In general, no marked differences were observed for the synthesized surfactants in comparison to BAC as a reference, for each analysed bacterium and the fungus *C. albicans*. Slightly lower selectivity indexes were calculated for Calu-3 cells, indicating a less specificity towards this cell line in comparison to Caco-2 cells.

The correlation analysis between MIC and CMC is shown in Figure 5. With the exception of *P. aeruginosa*, a very high positive correlation between these two parameters was found ($r \geq 0.929$; $p<0.01$) for all the tested species.

4. Discussion

Amino acid-based surfactants represent an interesting class of amphiphiles derived from natural sources, which have been extensively investigated in the last two decades [38] [39] [40]. Among them, those derived from cationic amino acids have been studied as alternatives to conventional antimicrobial compounds. Indeed, they display promising antibacterial and antifungal activities, together with good adsorption properties, as well as low toxicity and fast biodegradability [41,42]. Different chemical variations in the amphiphile structure have been explored, giving single chain, double chain or gemini surfactants with one or more cationic amino acid residues as polar head [41]. However, quaternary salts of amino acids esterified with fatty acids are not reported in the literature.

Although quaternary ammonium surfactants have a broad range of applications in different fields [43], their use in pharmaceutical formulations is limited due to their high haemolytic and irritant effect on mucosa [44–46]. Amino acid-based surfactants, prepared from different amino acids as polar head, have shown improved toxicological profiles on selected cell lines and surface active properties comparable to those observed for the most frequently used sodium dodecyl sulphate (SDS) [28,32]. The lower cytotoxic effect of amino acid-based surfactants can be attributed to the presence of a carboxylate group instead of a sulphate group in SDS. Nevertheless, the effect exerted by the presence of the amino acid on both the surface and biological properties of quaternary ammonium surfactants has not been investigated.

Surface and aggregation properties of the synthesized amino acid based-quaternary ammonium surfactants were studied here by tensiometry, conductivity measurements and dynamic light scattering in comparison to BAC as the reference compound. CMC values calculated for BAC were similar to those already reported in the literature [47,48]. Moreover, all calculated CMC values (for both BAC and the synthesized amino acid quaternary surfactants) were comparable between the two approaches (tensiometric and conductometric analyses). A statistically significant correlation was found between CMC from tensiometry and the EC_{50} values determined for both cell lines (Supplementary materials SF1 and SF2), suggesting that cytotoxicity is dependent on the aggregation behaviour, as already reported for anionic and non-ionic surfactants [28,49,50]. Although the relationship between hydrophobicity and cytotoxicity is predictable, this is the first time in which the correlation between EC_{50} and CMC values has been reported also for quaternary ammonium surfactants. EC_{50} values (from both MTS and LDH assay) were much lower than CMC

values, indicating that these amphiphiles show the ability of extracting and solubilizing phospholipids from mammalian epithelial cells, leading to the formation of mixed micelles and disruption of the cell membranes.

The most relevant biological aspect related to the quaternary ammonium compounds is the antimicrobial activity, widely exploited since 1930's for the formulation of disinfectants and antiseptics and more recently for the preparation of self-preserving biomaterials [13]. Quaternary ammonium surfactants possess a well-known broad spectrum of antimicrobial activity against Gram-positive and Gram-negative bacteria, as well as fungi and small enveloped viruses, but they are generally ineffective against spores and non-enveloped viruses. The antimicrobial effect of quaternary ammonium surfactants can be attributed to structural features of the molecules, which promote their insertion inside the cell membrane, thereby destabilizing the functionality of the membrane itself [51]. From previous studies, their antimicrobial activity was found to be dependent on the length of the alkyl chain linked to the *N*-group. Specifically, the maximum antimicrobial and/or antifungal effect was observed for the derivatives bearing a C12, C14 and C16 alkyl chain as a function of the different amphiphilic compound [13,52]. A linear relation between surface activity, mainly dependent on the length of the hydrophobic chain, and antimicrobial activity has been demonstrated for quaternary ammonium amphiphiles [53,54]. Amino acid-based quaternary ammonium surfactants showed a similar trend, since a higher activity in terms of MIC was observed for C12-C14 compared to C10 derivatives. From our results, it is also evident that bacterial species have different susceptibility towards the amino acid-based quaternary ammonium amphiphiles including BAC. Indeed, these materials showed a better antibacterial activity (as per the MIC values) against *S. aureus* and *E. faecalis* (Gram-positive) than against *E. coli* and *P. aeruginosa* (Gram-negative). This confirms the higher sensitivity of Gram-positive bacteria towards quaternary ammonium compounds [12,55] as also observed for other cationic amino acid based surfactants [41] and surface active cationic esters [56]. The lowest activity, for both BAC and amino acid-based quaternary ammonium amphiphiles, was found against *P. aeruginosa*, which can be explained by a different composition of bacterial wall in terms of fatty acids, lipopolysaccharides and outer membrane proteins acting as efflux pumps (specifically an overexpression of OprR) [57]. The differences observed in the spectrum of antibacterial activity have also been confirmed by considering the selectivity index (EC_{50}/MIC) since low ratios were obtained for Gram-negative bacteria (especially for *P. aeruginosa*), as a result of higher MIC values. Selectivity indices highlighted the different possible interactions of these amphiphiles with both mammalian and bacterial cell membrane. Altogether, the results emphasise the ability of these amphiphilic

molecules to combine effective surface properties with an attractive spectrum of antimicrobial activity, which could be advantageously exploited for a wide range of applications.

5. Conclusions

Quaternary ammonium amino acid-based surfactants showed physicochemical properties, cytotoxicity and antimicrobial activities that are strongly dependent on the length of the hydrocarbon chain and not noticeably affected by the amino acid (leucine or methionine) polar head. Derivatives with C12 and C14 hydrocarbon chains showed comparable cytotoxicity and antibacterial profiles, with the higher activity (lower MIC values) against Gram-positive bacteria compared to BAC, as reference compound. Thus, the synthesized amino acid-based surfactants can potentially find application as promising surface-active antimicrobials.

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Scheme 1 Synthetic approach for the preparation of leucine and methionine-based quaternary ammonium surfactants.

Figure 1 Surface tension *vs* concentration plot for the synthesized amino acid-based quaternary ammonium surfactants in comparison to BAC.

Figure 2 Conductivity (μS/cm) vs concentration for the synthesized amino acid-based quaternary ammonium surfactants in comparison to BAC.

Figure 3 Cell viability (%) of Caco-2 (A) and Calu-3 (B) cell lines after exposure to different concentrations of quaternary ammonium surfactants derived from leucine and methionine and the reference compound BAC. Dots represent the mean and bars the standard error (n = 4).

Figure 4 LDH release (%) from Caco-2 (A) and Calu-3 (B) cell lines after exposure to different concentrations of quaternary ammonium surfactants derived from leucine and methionine and the reference compound BAC. Dots represent the mean and bars the standard error (n = 3).

Figure 5 Correlation between MIC values (μM) determined on Gram-positive (*S. aureus*, *E. faecalis*), Gram-negative (*E.coli*, *P. aeruginosa*) and *Candida albicans* and CMC values (μM) calculated from tensiometry for the synthesized quaternary ammonium surfactants in comparison to BAC. The value of Pearson correlation coefficient (r) and its significance level (ns = $p>0.05$; * $0.01<p<0.05$; ** $0.001<p<0.01$; *** $p<0.001$) are reported on the upper left part of each panel.

Table 1 Surface tension parameters and critical micelle concentration (CMC) for the synthesized amino acid-based quaternary ammonium surfactants in comparison to BAC as determined by tensiometric and conductivity measurements. Values are the mean ± SD of three replicates.

	Tensiometry				Conductimetry
	CMC (mM)	γCMC (mN/m)	$10^6\Gamma_{max}$ (mol/m^2)	A_{min} (A^2)	CMC (mM)
BAC	1.02±0.06	31.16±0.05	5.35±0.27	31.08±1.60	0.86±0.11
TMOD LEU	2.92±0.45	30.14±1.68	6.08±0.13	27.33±0.58	2.29±0.24
TMOL LEU	0.62±0.06	31.27±1.49	5.98±0.47	27.64±0.97	0.51±0.13
TMOM LEU	0.36±0.02	31.58±0.18	5.11±0.88	32.40±1.55	0.28±0.12
TMOD MET	3.19±0.47	31.14±0.15	4.80±0.15	34.62±1.11	3.48±0.30
TMOL MET	0.73±0.12	30.94±0.95	4.08±0.20	40.74±1.23	0.52±0.10
TMOM MET	0.48±0.09	30.50±1.44	7.50±0.45	22.12±1.22	0.28±0.13

Table 2 Cytotoxicity parameter EC_{50} of the synthesized amino acid-based quaternary ammonium surfactants in comparison to BAC as determined by MTS and LDH assays. EC50 is the concentration of surfactants that caused 50% reduction of viable cells (MTT assay) or 50% release of LDH (LDH assay). Results are mean ± SD of at least three independent repeated experiments.

	MTS Assay (EC_{50} mM)		**LDH assay (EC_{50} mM)**	
	Caco-2	Calu-3	Caco-2	Calu-3
BAC	0.033±0.001	0.022±0.005	0.048±0.004	0.029±0.005
TMOD LEU	0.133±0.022	0.086±0.010	0.153±0.026	0.147±0.017
TMOD MET	0.186±0.040	0.190±0.023	0.221±0.052	0.273±0.051
TMOL LEU	0.031±0.013	0.016±0.001	0.039±0.012	0.017±0.002
TMOL MET	0.047±0.012	0.022±0.001	0.054±0.014	0.023±0.002
TMOM LEU	0.023±0.013	0.014±0.003	0.042±0.014	0.019±0.002
TMOM MET	0.024±0.012	0.015±0.003	0.048±0.080	0.019±0.001

Table 3 Calculated MIC values (mg/L) for the synthesized amino acid-based quaternary ammonium surfactants and the reference compound BAC. MIC values expressed as µM are reported in ST2.

	MIC values (mg/L)				
	S. aureus	*E. faecalis*	*E. coli*	*P. aeruginosa*	*C. albicans*
BAC	1	1	4	32	4
TMOD LEU	8	8-16	32	128	32
TMOD MET	16	8	32	128	32
TMOL LEU	1-2	1	8	32-64	4
TMOL MET	2	1	8-16	32	4
TMOM LEU	1	1-2	8-16	128	2
TMOM MET	1	1	8	64	2

Table 4 Selectivity index (EC_{50}/MIC) for the for the synthesized amino acid-based quaternary ammonium surfactants in comparison to BAC

	Selectivity index EC_{50}/MIC				
	Caco-2				
	S. aureus	*E. faecalis*	*E. coli*	*P. aeruginosa*	*C. albicans*
BAC	11.5	11.5	2.9	0.4	2.9
TMOD LEU	7.3	4.9	1.8	0.5	1.8
TMOD MET	5.3	10.7	2.7	0.7	2.7
TMOL LEU	9.7	14.6	1.8	0.3	3.6
TMOL MET	13.9	27.8	2.3	0.9	7.0
TMOM LEU	11.4	7.6	1.0	0.1	5.7
TMOM MET	12.4	12.4	1.5	0.2	6.2
	Calu-3				
BAC	7.7	7.7	1.9	0.2	1.9
TMOD LEU	4.7	3.2	1.2	0.3	1.2
TMOD MET	5.5	10.9	2.7	0.7	2.7
TMOL LEU	5.0	7.5	0.9	0.2	1.9
TMOL MET	5.4	10.7	0.9	0.3	2.7
TMOM LEU	6.9	4.7	0.6	0.1	3.5
TMOM MET	7.7	7.7	1.0	0.1	3.9

Highlights

Quaternary ammonium cationic surfactants derived from leucine and methionine were synthesized

Physicochemical properties, cytotoxicity and antimicrobial activities are dependent on the length of the hydrocarbon chain

Derivatives with a C12 or C14 hydrocarbon chain showed cytotoxicity and antibacterial profiles comparable to benzalkonium chloride

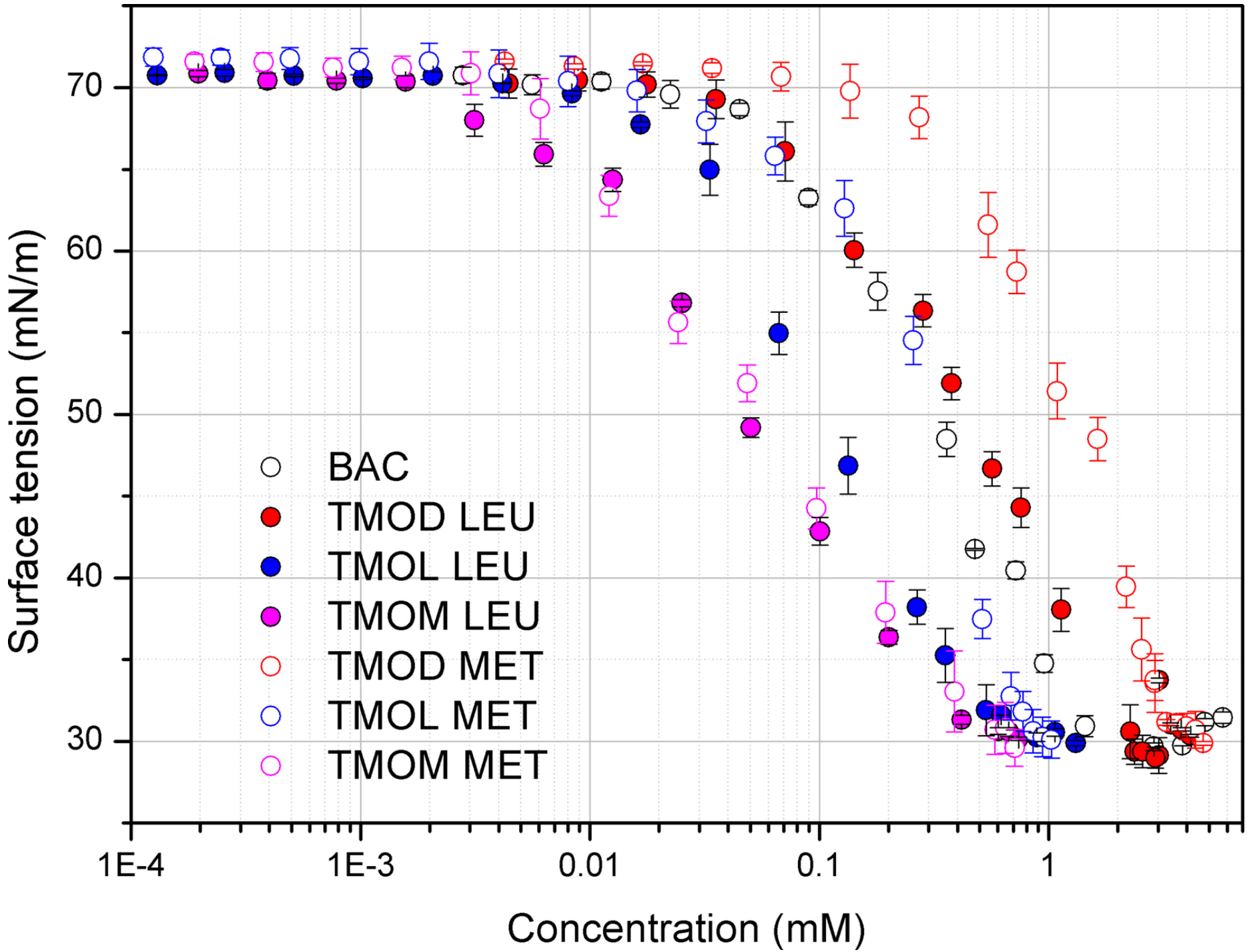

Surface tension (mN/m)
70
60
50
40
30
BAC
TMOD LEU
TMOL LEU
TMOM LEU
TMOD MET
TMOL MET
TMOM MET
1E-4
1E-3
0.01
0.1
1
Concentration (mM)

Figure 1

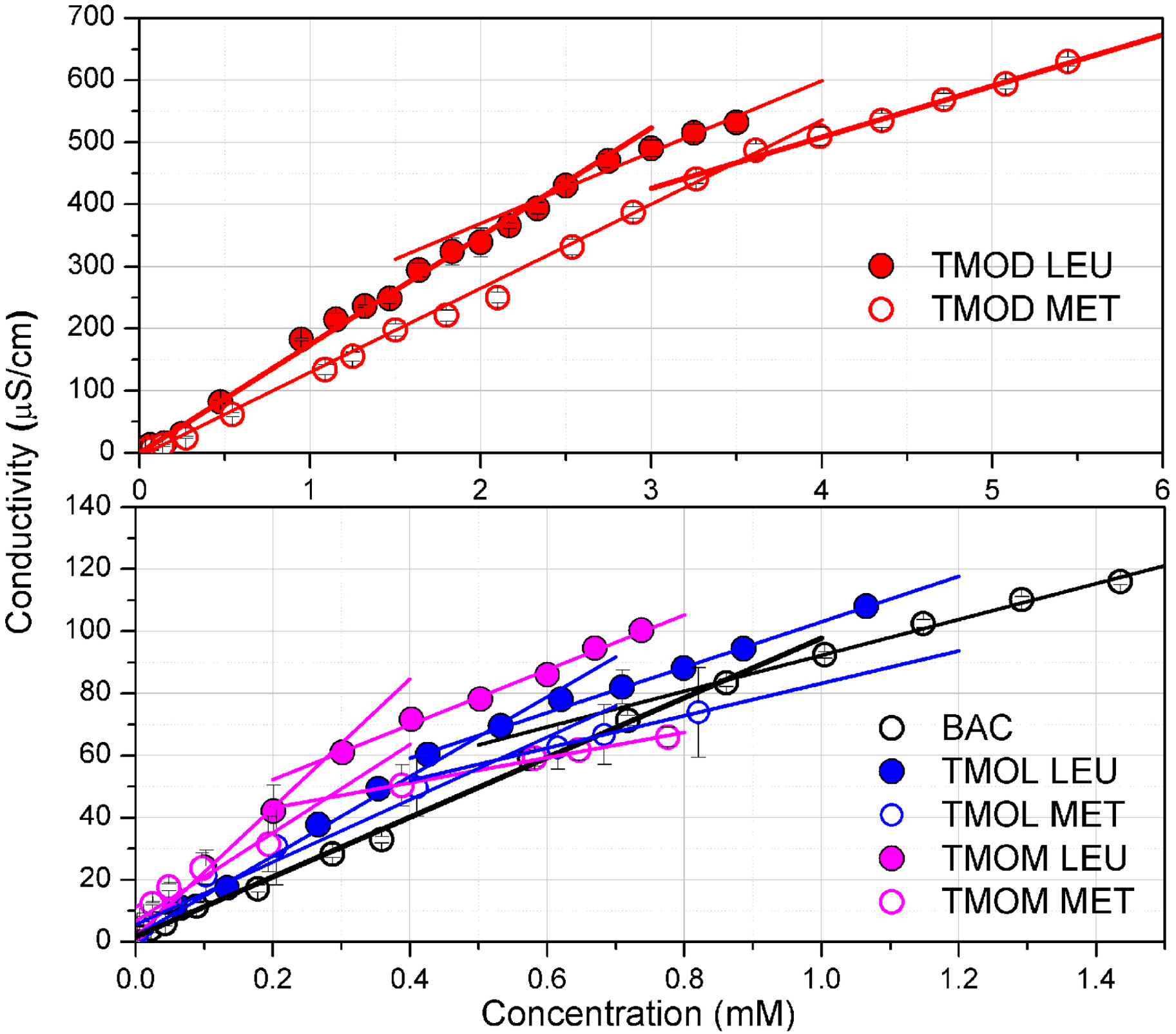

Conductivity (µS/cm)
Concentration (mM)
TMOD LEU
TMOD MET
BAC
TMOL LEU
TMOL MET
TMOM LEU
TMOM MET

Figure 2

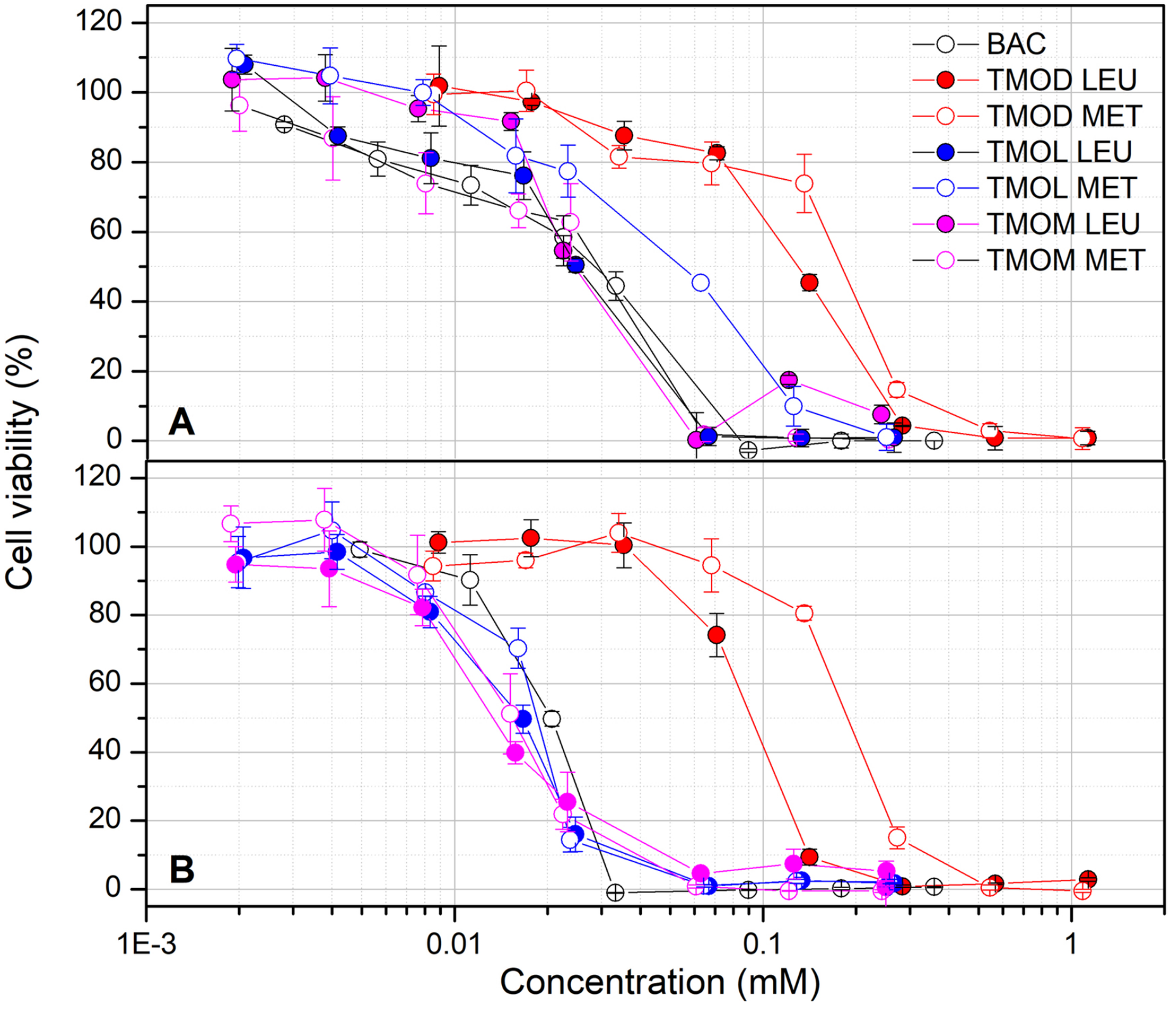

BAC
TMOD LEU
TMOD MET
TMOL LEU
TMOL MET
TMOM LEU
TMOM MET
A
B
Cell viability (%)
Concentration (mM)
120
100
80
60
40
20
0
1E-3
0.01
0.1
1

Figure 3

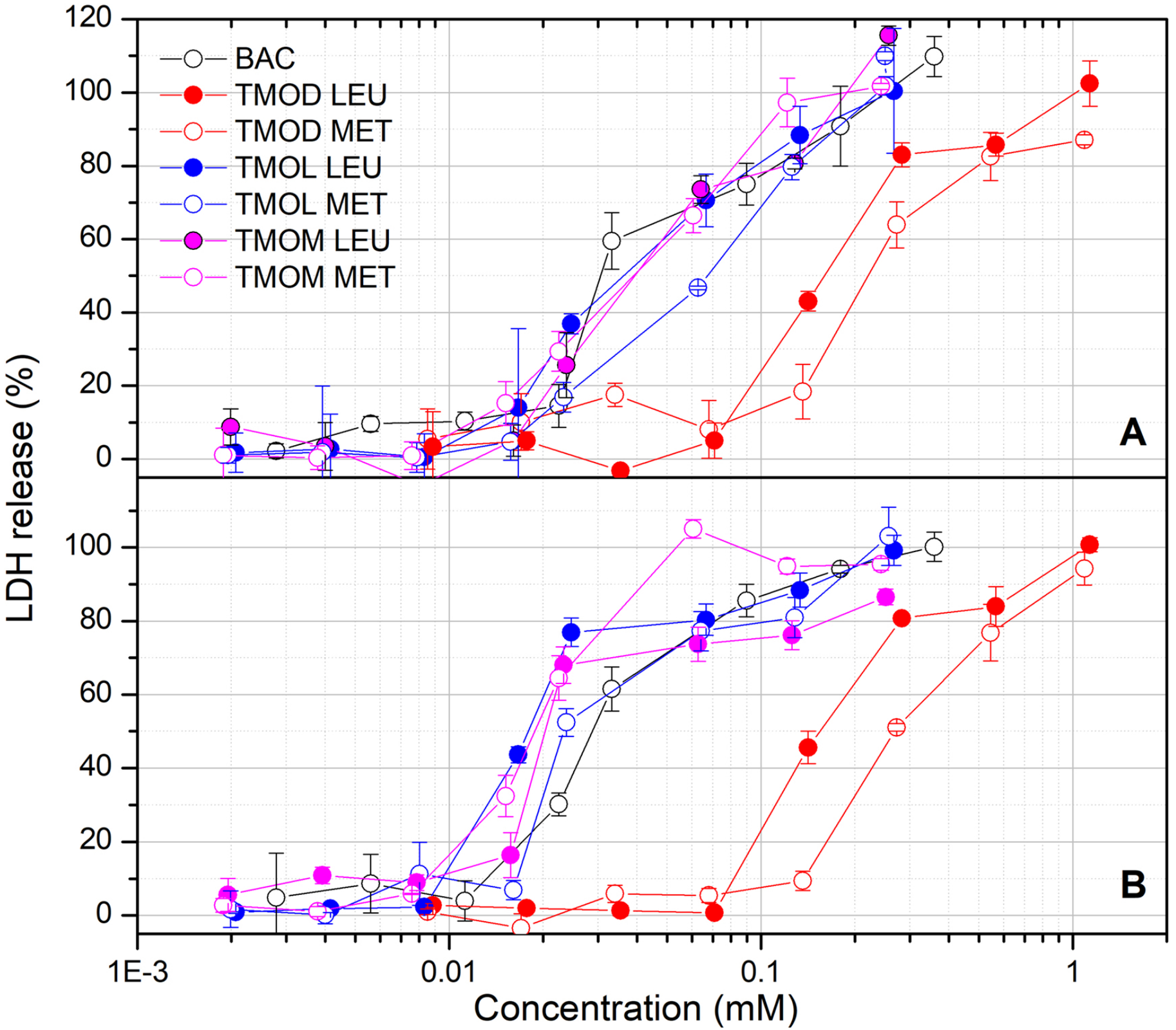
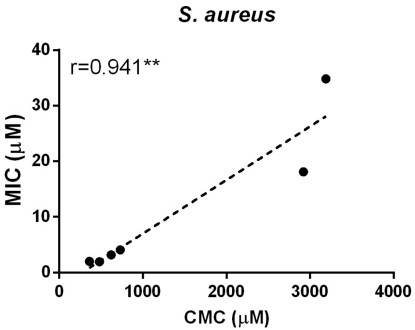
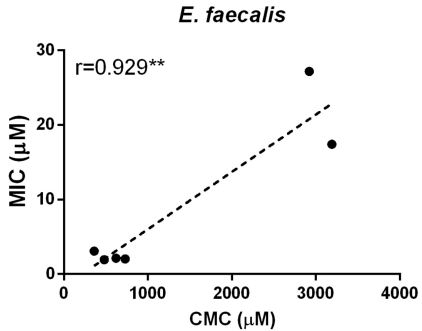
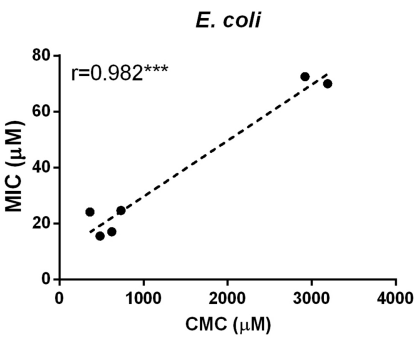
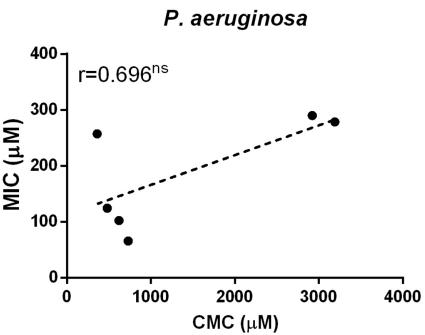

BAC
TMOD LEU
TMOD MET
TMOL LEU
TMOL MET
TMOM LEU
TMOM MET
LDH release (%)
Concentration (mM)
A
B
120
100
80
60
40
20
0
1E-3
0.01
0.1
1

Figure 4


S. aureus
r=0.941**
MIC (μM)
CMC (μM)
0
10
20
30
40
0
1000
2000
3000
4000


E. faecalis
r=0.929**
MIC (μM)
CMC (μM)
0
10
20
30
0
1000
2000
3000
4000


E. coli
r=0.982***
MIC (μM)
CMC (μM)
0
20
40
60
80
0
1000
2000
3000
4000


P. aeruginosa
r=0.696ns
MIC (μM)
CMC (μM)
0
100
200
300
400
0
1000
2000
3000
4000

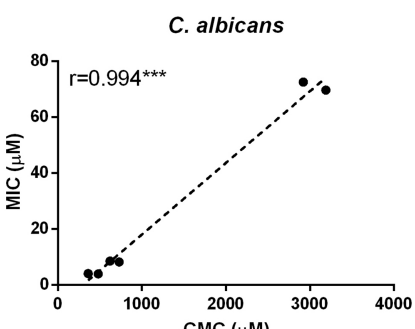
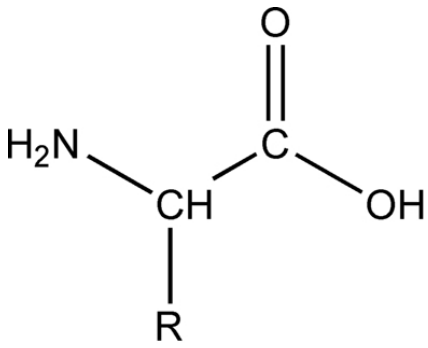
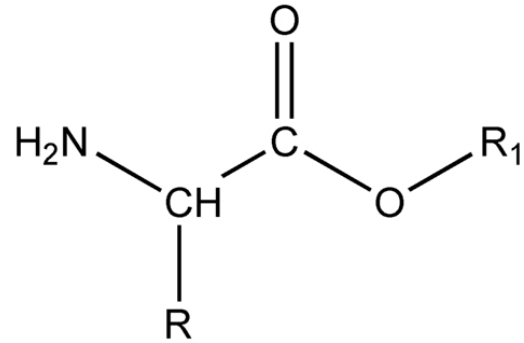

C. albicans
r=0.994***
MIC (μM)
CMC (μM)
0
20
40
60
80
0
1000
2000
3000
4000

Figure 5



Alchool; p-toluen sulphonic acid

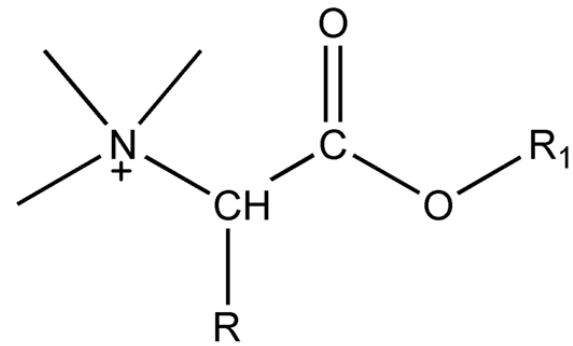
4h; anydrous toluene (reflux)



Iodomethane,
acetone,
24h

R: $-CH_3-CH-(CH_3)_2$ LEU
$-CH_2-CH_2-S-CH_3$ MET

R_1 $-CH_2-(CH_2)_8-CH_3$ TMOD
$-CH_2-(CH_2)_{10}-CH_3$ TMOL
$-CH_2-(CH_2)_{12}-CH_3$ TMOM



N,N,N-trimethyl-*O*-acyl amino acids

Figure 6